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The Present Status of the Transfer of Genetic Information and Its Control

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A. INTRODUCTION

The Cold Spring Harbor Symposium of two years ago dealt with many of the problems being considered at the present one. It is of interest to examine the advances which have been recorded in the intervening two years. The present paper focuses attention on the mechanisms of information transfer involved in the conversion of genotype into phenotype at the level of protein composition.

Evidence was presented in 1961 from a number of laboratories (Brenner, 1961; Gros et al., 1961b; Hurwitz et al., 1961; Jacob and Monod, 1961; Spiegelman, 1961) which led to what many accepted as a satisfactory molecular model of the intracellular flow of genetic information. The essential features are diagrammatically summarized in Fig. 1. Three principal modes of information

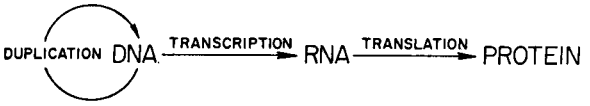

FIGURE 1. Flow of information from the genome.

transfer are recognized and distinguished by the end purposes they serve. The first is a duplication, designed primarily to provide exact copies for hereditary transmission. Here the same language and type existing in the original is used for copying. The second process is a transcription which generates complementary copies of RNA. In this step, the same language is employed, but there is conversion to a different type, analogous to transcribing from a handwritten to typescript. Finally, we have the third which involves a translational mechanism from the four unit language of the nucleic acids to the twenty element parlance of the proteins.

Any translational mechanism must of necessity involve the use of a dictionary. Excellent reasons can be advanced to identify the S-RNA molecules as components of the genetic dictionary, the others being the enzymes which recognize these RNA molecules and the corresponding amino acids. Further, since the translation results in a protein product, one must invoke the action of machines which generate the polypeptides. Ribosomes have been identified as important components of these protein synthesizing machines. Thus, both ribosomal RNA and S-RNA are functional constituents of the translational device.

This picture of information flow does not answer all questions concerning the use of genetic information. Nevertheless, it does help to delineate with clarity our areas of ignorance. In the first place, it must be recognized that the diagram of Fig. 1 describes primarily the sequence of events which ends in the ultimate translation of the genetic information into polypeptide chains. It is clearly not necessary to assume that all transcribed RNA is translatable into protein. Indeed we shall see that this is, in fact, not the case. An immediate question is the origin of the two RNA types which function at the translational step. Also left unanswered is the nature of the mechanisms controlling transcription with respect to the size distribution of RNA messages generated, the conditions under which particular sections of the DNA are transcribed, the problem of whether one or both of the strands of the DNA are transcribed, etc. Further, some issues which are now of central interest did not arise in the 1961 Symposium. One is the question of control at the point of translation. Another concerns the problems posed by RNA genomes and the mechanisms they employ for replication and transmission of their information for purposes of protein synthesis.

We would like to summarize what we can now say about these unresolved problems. For ease of discussion, we may list them in the order in which they will be considered as follows.

B. The origin of ribosomal RNA.

C. The origin of the translational S-RNA.

D. The size distribution of RNA messages and its significance for "operon" operation.

E. Control of order and frequency in the translation of polycistronic messages into protein.

F. Random vs. non-random transcription of a DNA genome.

G. The relation of inducers of enzyme synthesis to transcription.

H. Do one or both strands of the DNA generate genetic messages?

A number of laboratories have made significant contributions toward the resolution of one or more of these problems and their representatives will undoubtedly discuss their data at length. In the present description of experiments relevant to the issues listed, greater emphasis on detail will be given to those performed in our own laboratory. (We will have occasion in the following discussion to make repeated references to DNA-dependent-RNA polymerase; RNA-dependent-RNA-polymerase etc. For brevity and alliterative usefulness we will use the following terms. The DNA-dependent-DNA-polymerase which produces DNA duplicates will be called a *duplicase*. The RNA-dependent-RNA-polymerase which replicates RNA will be called a *replicase*. The transcribing DNA-dependent-RNA-polymerase which produces RNA complements will be referred to as a *transcriptase*.)

B. THE ORIGIN OF RIBOSOMAL RNA

Despite the fact that ribosomal RNA constitutes the bulk (85% of the cellular RNA) its mode of origin was little understood at the time of the 1961 Symposium. While clearly not exhaustive, two alternatives could be entertained. One would assume a DNA dependent reaction and the other would involve a synthetic mechanism independent of DNA. The fact that the base composition of ribosomal RNA shows no tendency to correlate (Woese, 1961; Spiegelman, 1961) with homologous DNA is irrelevant to a choice between the two hypotheses. The presumed DNA segment involved might be so small as to constitute a statistically inadequate sample of the overall base composition.

Posing the problem in the form of these two alternatives suggests the following question, pertinent to a decision and amenable to experimental resolution. *Does DNA contain a sequence complementary to homologous ribosomal RNA?* An approach to questions of complementarity is, in principle, provided by Hall and Spiegelman's (1961) demonstration of specific hybrid formation between T2-DNA and the RNA synthesized in *E. coli* infected with T2. These experiments used differently labeled nucleic acids for ease and sensitivity of identification of hybrid. They also took advantage of equilibrium density gradient centrifugation in swinging bucket rotors to separate free RNA from the DNA-RNA hybrid complex.

The technical difficulties inherent in using the hybridization test to establish the existence of complementarity between ribosomal RNA and some sequence of the DNA had already been discussed (Spiegelman, 1961) in the 1961 Symposium. The major complications stem from the numerology of the situation. For example, the 23 S ribosomal RNA component is 1.1×10^6 in molecular weight, so that even if a specific complex were formed, it might involve only 0.02% of the DNA available in the genome of *E. coli*. We were, therefore, faced with the problem of designing experiments sufficiently sensitive to detect hybridization at this level. This can theoretically be accomplished by raising the specific activity of the RNA used in the hybridization to suitable levels. Indeed, in principle, the test could be made definitive in both a positive and a negative sense. However, magnification of the sensitivity of hybrid detection by these means carries with it the attendant danger that complexes might be observed which are irrelevant to the question being asked. Apparent hybrids might represent any one of the following: (1) complexes between DNA and small amounts of complementary RNA contaminating the ribosomal preparations, (2) mechanical trapping of small amounts of ribosomal RNA in the strands of DNA, (3) partial hybridization resulting from accidental coincidences in complementarity of short sequences.

In view of these possible complications, observations of labeled RNA accompanying the DNA in a density gradient must be supplemented with independent information which establishes that the RNA, so complexed, is ribosomal and that it is specifically hybridized to the DNA. Experimental procedures which provide the requisite information were devised by Yankofsky and Spiegelman (1962a). A central device depended on the resistance of hybrid to nucleolytic enzymes for differentiating between chance pairing in restricted regions and extensive specific hybridization.

The experiments performed succeeded in establishing the existence of sequences in *E. coli* DNA complementary to its ribosomal RNA. Proof depended on showing that a hybrid complex, resistant to ribonuclease, is specifically formed only with homologous DNA.

The fact that specific pairing of ribosomal RNA with complementary sequences in homologous DNA led to the following two predictions: (1) the ratio of RNA to DNA in a specific complex should approach a maximum value at levels indicating the involvement of a minor fraction of the DNA, yielding the percentage of the genome concerned with ribosomal RNA synthesis. (2) Non-ribosomal RNA should not compete for the same site. These predictions were confirmed (Yankofsky and Spiegelman, 1962b). Saturation experiments with 23 S RNA suggested that the corresponding complementary region corresponds to between 0.1 and 0.2% of the total genome. Competition for

the ribosomal sequence in DNA was tested by using two identifying radioactive isotopes. Tritium labeled non-ribosomal RNA from the same organism was shown to hybridize with the DNA without displacing P^{32}-labeled ribosomal RNA from the hybrid structure. Therefore, the two types do not compete for the same sequences in the DNA.

The amount of ribosomal RNA complexed per unit of DNA at saturation suggests that several repeating, similar or identical, sequences for the 23 S RNA exist. Further, the density shift of the hybrid suggested that these units are not scattered but contiguous in the DNA structure.

The experiments just summarized were carried out with the 23 S ribosomal RNA and left unanswered the relation of these findings to the 16 S RNA ribosomal component. The similarity in base composition and the fact that the molecular weights of the 23 S and 16 S are almost in the relation of 2:1 suggested the possibility of a common origin, the 23 S perhaps being the dimer of the 16 S. Definitive evidence on whether or not they derive from the same sequence can be readily obtained by the hybridizing technique combined with the double-labeled experiments already described.

The following sorts of information were pertinent to resolution.

(1) *Saturation plateaus.* If the 16 S and 23 S are derived from the same sequence, the RNA to DNA ratio found in the hybrid at saturation should be the same for each of the two types of RNA.

(2) *Additivity.* At the saturation RNA to DNA ratio of either, the addition of the other should lead to no further complex formation if they are derived from the same sequences. If, on the other hand, the sequences of origin are different, additional hybrids should be observed.

(3) *Competitive interaction.* By the use of two identifying isotopic labels, the presence or absence of competition during hybridization can be established. Absence of competitive interaction would indicate distinct sequences and its existence would argue for identity.

Experiments testing for these various possibilities were carried out (Yankofsky and Spiegelman, 1963) with RNA derived from *Bacillus megaterium.* The data obtained established that; (1) the maximum amount of RNA which can hybridize per unit of DNA is different for the two types of ribosomal RNA; (2) at saturation concentrations of each, the amount of hybrid formed is additive when 16 S and 23 S RNA are both present; (3) no evidence of competitive interaction between the two types of RNA for the same sites can be detected.

All these findings are difficult to reconcile with a common origin. It may, therefore, be concluded that 16 S and 23 S ribosomal RNA are derived from DNA sequences unique to each. In conclusion, we may state that the use of the hybridizing procedure developed as a test for sequence complementarity, coupled with a number of necessary auxiliary devices, provided an answer to the question of the ultimate origin of ribosomal RNA. It was possible to demonstrate that DNA contains sequences capable of specifically hybridizing with homologous ribosomal RNA. It was further possible to show that the 23 S and 16 S ribosomal RNA components originate from different sequences in the DNA.

From such data we may tentatively conclude that ribosomal RNA is also made by a DNA dependent mechanism. These observations do not, however, eliminate the possibility that this is augmented by a DNA independent synthesis.

C. THE ORIGIN OF THE TRANSLATIONAL S-RNA

There are excellent reasons (Nirenberg et al., 1962; Chapeville et al., 1962) for identifying the soluble ribonucleic acid (S-RNA) molecules as components of the genetic dictionary which permits translation from the nucleic acids into polypeptides. It was of obvious interest to determine the relation between these RNA molecules to the genome. Again one would, in essence, like to know whether sequences exist in homologous DNA which are complementary to those which occur in the S-RNA molecules. The basic techniques employed to settle this question in the case of the ribosomal RNA were applicable, with only slight modifications, to the same issue with respect to the S-RNA. Two groups (Giacomoni and Spiegelman, 1962; and Goodman and Rich, 1962) simultaneously carried out very similar investigations and came up with completely concordant results. Experiments were performed which established that specific complexes could indeed be exhibited between S-RNA molecules and homologous DNA. The nature of the data provided can be listed as follows.

(1) It was shown directly that the RNA found to be complexed with the DNA in a cesium chloride density gradient centrifugation was indeed S-RNA by a direct analysis of the base composition of the hybridized material.

(2) It was demonstrated that the RNA so hybridized is much more resistant to degradation by ribonuclease than is a free RNA control.

(3) The S-RNA was found to saturate the DNA at levels indicating that only a small proportion (approximating 0.023%) of the DNA is complementary to the S-RNA molecules.

(4) Mixtures containing two heterologous S-RNA preparations, each with an identifying isotopic

label, and a DNA homologous to one were hybridized. Complex formation occurred only between the homologous pair. Converse experiments involving two different DNAs and one S-RNA preparation confirmed the specificity of hybridizations (Giacomoni and Spiegelman, 1962).

The fact that S-RNA saturates the DNA at about 0.023% is consistent with what would be expected if the genetic code were degenerate. Despite the complicated operations required to obtain this number, it is remarkably reproducible, and was obtained by both groups. Therefore, the value can be given some credence. If anything, it may perhaps be an underestimate since conditions for hybridization may not be optimal and the treatment with ribonuclease may slowly remove some S-RNA that had entered into a true complex.

The expected saturation level can be estimated from the molecular weight equivalent to the genome of *Escherichia coli* and the number of different kinds of S-RNA molecules, each of which has a molecular weight of 2.5×10^4. If the genetic code is not degenerate, 20 different S-RNA molecules would be expected. If the code is degenerate, more than 20 will be needed in the dictionary. The plateau predicted by the non-degenerate case is 0.01%. The fact that it is at least twice as high suggests that some amino acids are identified with more than one S-RNA molecule.

The data available suggests that the genetic dictionary is universal, or nearly so. However, the coding triplets probably occupy only a small proportion of the S-RNA strands. Although the function of the non-coding stretches of approximately seventy nucleotides is as yet unknown, they provide an opportunity for biological individuality by sequence variation without disturbing the translational function of the S-RNA. The specificity of complex formation exhibited between S-RNA and homologous DNA shows that this opportunity for individualization was not neglected in the course of biologic evolution. Thus, although the S-RNA of *E. coli* can translate the genetic message of a rabbit into hemoglobin, (von Ehrenstein and Lipmann, 1961) this S-RNA can be uniquely identified with the genome of its origin.

Ribosomal RNA appears to have the same combination of genetic uniqueness and uses unrestricted by specificity requirements. Ribosomes are comparatively indifferent to the origin of the genetic messages to which they respond, (Martin et al., 1962; Speyer et al., 1962; Berg and Lagerkvist, 1962). However, their sequences are unique since they hybridize readily only to homologous DNA. One other feature is shared by these two molecular species. Although their sequences vary, the over all base composition is remarkably similar in a variety of organisms. Thus, the base composition of ribosomal RNA from *Pseudomonas aeruginosa*, with a DNA containing 64% guanine-cytosine (G, C) is indistinguishable from that of *Bacillus megaterium*, the DNA of which contains 44% (G, C).

We are faced with a paradox that two sets of cistrons—those for S-RNA and ribosomal RNA, have resisted the drift toward different average base compositions. A possible answer may reside in the fact that both of these types of RNA must interact with protein. The ribosomal RNA must be assembled into a defined structure along with a variety of different proteins which are coded by other sequences. Any changes in the ribosomal RNA which are structurally incompatible, and which are not compensated by appropriate changes in the cistrons coding for the ribosomal proteins, would be eliminated. Similarly, the S-RNA molecules must interact with the activating enzymes. Again, any change in them which leads to a lowered efficiency of recognition by the homologous activating enzyme would be severely selected against.

The results described in this section and the preceding one establish that all recognized RNA components, including translatable genetic messages, the non-translatable ribosomal RNA, and the translational S-RNA have corresponding complementary sequences in homologous DNA. Their formation can, therefore, adequately be explained on the basis of the DNA dependent RNA synthesizing system.

D. THE SIZE DISTRIBUTION OF RNA MESSAGES AND ITS SIGNIFICANCE FOR "OPERON" OPERATION

The study of the physical properties of RNA messages was initiated by Nomura, Hall, and Spiegelman (1960) in their attempt to provide physical evidence for the existence of the T2-specific RNA discovered by Volkin and Astrachan (1956). These early studies employed a preliminary separation of the ribosomes by the usual two to three hr centrifugations at 40,000 rpm. A determination of the size distribution of the pulse-labeled RNA found in such preparations from T2 infected cells revealed a rather broad distribution centering between 8–14 S. Other laboratories (Gros et al., 1961a, b; Brenner et al., 1961; Tissieres and Hopkins, 1961), seeking to extend these observations to uninfected cells, used the same methods of separation and, not too surprisingly, found the same size range. In addition, complementary RNA synthesized in vitro by the DNA dependent RNA polymerase was found by Geiduschek et al., (1961)

to have an average sedimentation constant of about 6 S.

In view of the apparent unanimity of these observations, it is perhaps understandable that in the 1961 Symposium references to "messenger" RNA implied that a distinct average size of 8 to 14 S is a characteristic which appears to distinguish this RNA from the three major components (23 S, 16 S, and 4 S) of the cell. However, it was pointed out (Spiegelman, 1961) in describing the "stepdown" transition experiments (Hayashi and Spiegelman, 1961) that we had reasons to doubt that this was, in fact, the case. As our own experience with complementary RNA accumulated, it had become evident that this type of RNA was much more polydisperse and uniquely susceptible to degradation in extracts. It seemed possible that the small, comparatively homogenous size observed in the early experiments might be a consequence of breakdown in the course of preparation.

Our own ideas about size were revised in the course of a study by Hayashi and Spiegelman (1961) designed to find conditions in normal cells which would suppress ribosomal RNA formation and permit the selective synthesis of the message fraction. "Step-down" transitions from "complete" to synthetic medium were found to be the most suitable. In these experiments the procedure employed for RNA isolation avoided prolonged contact with enzymatically active extracts. The result was that a much wider (30 S–8 S) distribution of size ranges in the message fraction was observed.

These findings with normal cells made it desirable to reinvestigate T2-specific RNA using the same precautions. The results obtained (Sagik et al., 1962) were now in agreement, the same wide diversity from above 30 S to 8 S being observed. Similar findings were soon reported with other systems (Otaka et al., 1962; Monier et al., 1962; Hiatt, 1962).

It was naturally of some interest to provide an interpretation of this size diversity and particular interest centers, quite naturally, on the large message fraction. In discussing this question in the Rutger's Symposium, Spiegelman (1963) proposed an explanation in terms of "operon" function. The basic argument may be briefly repeated, since it is of interest for the experiments to be described in the following section. It seems reasonable to suppose that the molecular weights of the basic subunits of most proteins will be found between 10,000 and 40,000. If the coding ratio is three and if each RNA message codes for only one protein, message RNA molecules much greater than 14 S are unexpected. The fact that they do occur implies that some messages code for several proteins. These would be expected to arise from the continuous transcription of contiguous cistrons. A simple physical basis is thereby provided for the "operon" concept which emerged from the brilliant theoretical analysis of Jacob and Monod (1961). According to these authors, two cistrons are in the same "operon" if they are controlled by the same "operator" gene. We extended this to say that if two cistrons belong to the same operon, they are likely to be transcribed into the same continuous molecule of RNA. We have thus the production of what may be called "polycistronic messages."

A number of consequences of great experimental interest follow from the considerations just recorded. Thus, the genetic punctuation which separates "operons" is designed to interrupt the transcription process and will be confined to the DNA. The genetic punctuation which separates cistrons is a device which interrupts the translational mechanism and should be found both in the DNA and in polycistronic RNA messages. Finally, there is the implication of a control mechanism at the point of translation to determine frequency with which each cistron is used. A device of this sort would avoid the synthesis of precisely the same number of molecules of every protein coded in a given polycistronic message. We now turn our attention to an experimental analysis of this last implication. (Additional evidence for polycistronic messages was presented at the present symposium and similar consequences for translational controls were drawn. See, in particular, B. Ames and P. Hartman; R. G. Martin; B. Guttman and A. Novick; I. Zabin; N. Zinder (in discussion by T. P. Bennett following Yamane, Sueoka and Cheng).

E. CONTROL OF ORDER AND FREQUENCY IN THE TRANSLATION OF POLYCISTRONIC MESSAGES INTO PROTEIN

We now consider the problem of whether one can provide experimental evidence for order and frequency control in the translation of polycistronic messages. For reasons which will become obvious, our attention was turned to the RNA viruses.

The RNA viruses virtually guarantee the existence of polycistronic molecules and they also provide an ideal source of a uniform population of RNA molecules each of which codes for the same limited number of proteins. The reasons for asserting the polycistronic nature of viral RNA may be briefly stated. In the first place, mutational data (Wittman, 1961) with tobacco mosaic virus establishes that viral RNA determines the amino acid composition of the coat protein. Further, it can be demonstrated (see Spiegelman and Doi, this volume and Horuna et al., 1963) that viral RNA

replication must involve a new type of RNA-dependent-RNA-polymerase which, as noted in the Introduction, we will refer to as a "replicase." Since this new enzyme must be synthesized before RNA duplicates can appear, the incoming viral RNA must obviously contain the information necessary for the production of the "replicase." Thus, all viral RNA molecules must contain at least two cistrons and are, therefore, polycistronic.

The fact that the viral RNA codes for both coat protein and the RNA "replicase" implies that the RNA molecule must either contain, or respond to, a device which dictates the number of times a particular cistron is translated. This follows as a numerical consequence of the number of coat protein subunits required to complete a virus particle compared with other proteins specifically necessary for mature virus formation. To illustrate the reasoning by a specific case, we take as our example the RNA bacteriophage MSϕ2 which is the object of the experiments to be described here. This RNA is approximately 8×10^5 in molecular weight (Doi and Spiegelman, in preparation) and, as will be evident from the data to be detailed, codes for at least 3 proteins. The molecular weight of the coat protein subunit is about 20,000 and there are approximately 200 such subunits per particle. Since the normal burst size of this virus is between 1,000 and 10,000 per cell, it is evident that between 2×10^5 and 2×10^6 coat protein molecules are made per cell per lytic cycle. It is difficult to imagine that an equal number of replicase molecules are also synthesized. The same argument would evidently apply to any other enzyme or protein which is not included as a component of the mature particles. (See also A. Rich and discussion by T. P. Bennett following Yamane et al., in this volume, for a similar conclusion.)

The situation described virtually demands a mechanism which insures that the cistron for the coat protein be translated more times than those which correspond with some of the others. It seems likely that this device would be built into the RNA molecule and can operate even outside the cell. Experiments were, therefore, undertaken by Ohtaka and Spiegelman (1963) to see whether evidence for a control mechanism of this sort can be detected in an in vitro system. Clearly, one requirement is the ability to distinguish the coat protein from the others. This possibility is provided by the RNA bacteriophage MSϕ2, since its coat protein lacks histidine (Doi and Spiegelman, in preparation). Consequently, histidine can be employed as a marker to detect the appearance of histidine containing proteins synthesized under the direction of the viral RNA. With this device, one can readily compare the kinetic details of the appearance of coat and non-coat proteins.

Nathans et al., (1962), using the system of Nirenberg and Matthaei (1961), showed that the RNA of F2 stimulates the incorporation of amino acids into proteins which are similar in their peptide fingerprint pattern to the coat protein. Further, proteins which contain histidine are also formed. We have confirmed these findings with the RNA isolated from MSϕ2.

The situation described permits the design of a comparatively simple experiment which can, in principle, provide definitive information on the presence or absence of order and frequency regulation in the synthesis of the proteins directed by the viral RNA. The kinetics and time of entry into protein of histidine are compared with that of some other amino acids commonly contained in all the proteins synthesized. Before using the system for the purposes indicated, certain aspects must be carefully controlled.

In a comparison of the incorporation of histidine with some other amino acids, a number of precautions must be taken if meaningful data are to be obtained. In the first place, irrelevant kinetic differences, which might occur from one preparation to another, must be avoided. This can be achieved by using two different isotopes, for example, H^3 and C^{14} to identify the two amino acids being compared. The incorporation of both in the same reaction mixture can then be followed by measuring radioactivity in a liquid scintillation spectrometer and thus each amino acid serves as an internal control of the other. Under these circumstances, observed kinetic differences can be accepted as real and interpretable providing other controls are also run. Further, the specific activities of the amino acids used must be adjusted to the extent of their incorporation so that the sensitivities of the observations of the two are comparable. Finally, background activity, unassociated with synthesis specific to that directed by the viral RNA, must be eliminated by pre-incubation of the ribosomal preparation with all necessary supplements prior to adding the viral RNA and the labeled amino acid.

Because of its central importance in use of the system, it was necessary to check directly that the viral RNA is actually serving as a polycistronic message in the in vitro system being employed. The conclusion that this is the case would receive direct support by the demonstration that histidine is incorporated into a protein or proteins separable from the coat protein. The insolubility of the coat protein in the usual buffers, and the tight ribosomal association of the histidine containing proteins synthesized, presented difficulties which had to be

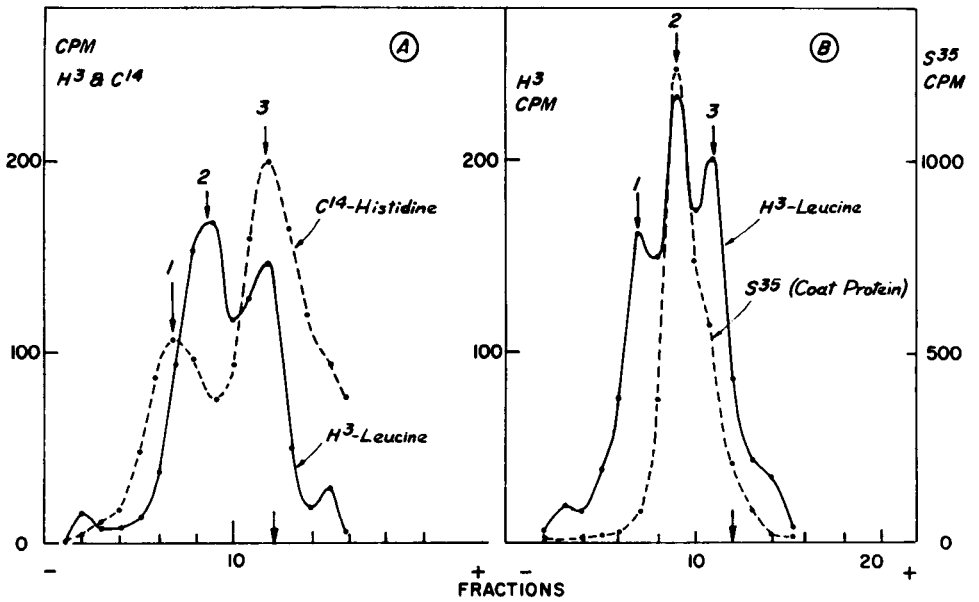

FIGURE 2. Electrophoretic separation of components synthesized under influence of viral RNA (Ohtaka and Spiegelman, 1963).

(A) The incubation volume is 0.5 ml. After the usual 20 min incubation, H^3-leucine (final specific activity 2.8 mc/μM) and C^{14}-histidine (at 22 μc/μM) along with MSϕ2-RNA (160 μg/ml) were added. After 60 min, 0.3 ml of the reaction mixture was adjusted to 5 M urea, 0.01 M EDTA and 0.3 M Na acetate at pH 5.6. The material was then applied at the position indicated by the arrow to a 2 × 36 cm column of 40 g of "geon" (B. F. Goodrich Chemical Co.). Electrophoresis in 0.03 M Na acetate, pH 5.6; 5 M urea; 0.01 M EDTA was then instituted at 500 V and 10 mAmp. After 24 hr, 2 cm sections were cut out and eluted with 3 ml of the urea-EDTA-acetate buffer. Carrier protein (1 mg serum albumin) was added to each fraction. Labeled material was precipitated with 6% TCA containing 0.5% phosphotungstic acid, washed on millipore filters, and counted in a liquid scintillation spectrometer.

(B) Everything is as described in 2A except that labeling was only with H^3-leucine, and S^{35}-labeled coat protein was added to the mixture prior to application to column. The S^{35}-labeled virus particles were prepared as described previously (Doi and Spiegelman, 1962) for P^{32} labeling except that 7 mc of S^{35} as $SO_4^=$ was included, the $MgSO_4$ in the medium being adjusted to 0.001 M. The phage protein was purified by the acetic acid procedure (Fraenkel-Conrat, 1957).

overcome. It was eventually found that the addition of urea (5 M) and versene (0.01 M) solubilized all components, permitting a successful separation of the synthesized proteins by electrophoresis on columns of geon. A typical outcome is given in Fig. 2A where the reaction mixture was allowed to incorporate C^{14}-histidine and H^3-leucine for 60 min. It is clear from the profiles of Fig. 2A that the three major components indicated by the numbers 1, 2, and 3 are readily identifiable. The relative distributions of the histidine and leucine suggest that component 2 is either low in, or completely lacks, histidine and is to be identified with the coat protein. That this is the case is strongly supported by the results of the experiment described in Fig. 2B. Here, the product of a reaction mixture labeled for 60 min with H^3-leucine was mixed with S^{35} labeled protein purified from isolated viral particles. The resulting mixture was then subjected to electrophoretic separation. It is clear that the S^{35} labeled protein moves into the same position as peak 2. These results would appear to establish that MSϕ2 viral RNA is polycistronic in the system being observed and contains cistrons which code for at least three protein components. Two of the components clearly contain histidine and the third is identifiable with the coat protein which lacks this amino acid.

There is too much contamination of one peak by another in Fig. 2A to permit a meaningful approximation of the relative amounts of each component formed. An estimate can, however, be obtained from the comparison of the molar amounts of histidine incorporated to that of some other amino acid. In a typical reaction run for 60 min, 0.77 mμmole of valine and 0.02 mμmole of histidine were incorporated. Thus, for every 38 moles of valine, only one mole of histidine is employed. On the basis of the 6:1 ratio of valine to histidine commonly found in proteins, one would estimate that the ratio of the masses in peaks 1, 2, and 3 are approximately as 1:12:1. If the comparison is made in terms of number of molecules, the ratio is likely to increase further in favor of the coat protein, in view of its very small molecular weight. In any case, the data indicate preferential synthesis of a protein which does not contain histidine.

With the information described available, one can employ the system to analyze the questions of randomness and frequency by comparing the

kinetics of entry of histidine with some other amino acid.

In thinking about possible outcomes, one must recognize that order versus randomness in the translation in the set of cistrons involves questions of times, frequency, and the relation of these to location in the RNA molecule. Thus, consider the coat protein cistron and two others arranged in some sequence. The following possibilities can be imagined.

(1) There is no preferred order of translation and the frequency of translation is equivalent for all three cistrons.

(2) There is a preferred order and the translation frequency is equivalent for all three cistrons.

(3) There is no preferred order, but the number of translations per unit time is considerably greater for the coat protein.

(4) There is a preferred order and the translation frequency of the coat protein is considerably higher than the others.

We have already noted that mechanisms similar to 1 and 2 are unlikely in the cell, since they predict equal numbers of all three types of protein molecules would be synthesized per unit time. They could, however, occur in a cell-free system. A distinction between these two mechanisms can be achieved only by limiting the experiment to a time interval comparable to one translation of each of the cistrons. In any long term experiment both would anticipate similar kinetics of appearance for all three proteins. The third mechanism would predict that histidine-containing proteins would begin appearing at the same time as the non-histidine-containing coat protein. However, the relative rate of formation of the latter would be higher, and remain constantly so throughout the period of active synthesis. Finally, the fourth mechanism would predict a break in the kinetics of appearance of the histidine-containing proteins. The position of the break would depend on the temporal order in which the cistrons become active.

A typical experiment comparing the kinetics of entry into protein of H^3-histidine and C^{14}-valine is described in Fig. 3A. There is clearly no detectable lag in the time of entry of C^{14}-valine into hot acid-precipitable protein. It is equally evident that there is a distinct lag, amounting to about 6 min, before the appearance of proteins containing H^3-histidine. Similar experiments were performed with other amino acids and with the radioactive

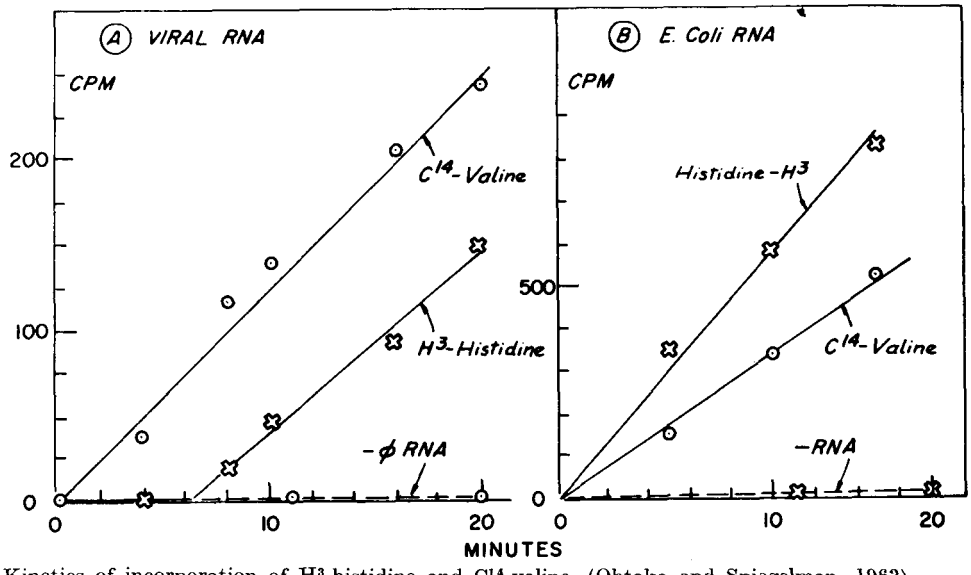

FIGURE 3. Kinetics of incorporation of H^3-histidine and C^{14}-valine. (Ohtaka and Spiegelman, 1963).

(A) The reaction mixture (1.0 ml) is as detailed in first section of Fig. 2. Following the usual 20 min preincubation, H^3-histidine (final specific activity 1.1 mc/μM) and C^{14}-valine (final specific activity 8 μc/μM) were introduced. At the same time, 160 μg of RNA purified from MSϕ2 was added to one and omitted from a control. The specific activities chosen were determined by the proportion of the two amino acids incorporated and the counting efficiencies of C^{14} and H^3. The points represent the cpm (H^3 or C^{14}) found in each 0.1 ml removed at the indicated time intervals. Only the C^{14} counts are shown in the control lacking RNA since the H^3 counts were equally negligible. Each sample was prepared for counting as described in Fig. 2. Window settings for double counting were such that cross channel contamination was 25% in the direction of C^{14} to H^3 and 5% in the reverse direction. The same settings yielded similar results with S^{35} and H^3.

(B) All details are as described above in 3A. Here, however, *E. coli* RNA (1.6 mg) was added. To increase the proportion of the message fraction, log phase cells were incubated in growth medium for 30 min in the presence of chloramphenicol (50 μg/ml). This has consistently resulted in RNA preparations extremely active in stimulating incorporation of amino acids. Again a control was run without added RNA. As shown, negligible amounts of histidine were incorporated and the same was true for valine.

labels reversed; for example, H^3-leucine and C^{14}-histidine. Again, there was a 5 to 6 min lag in the incorporation of histidine and none with leucine. The lag cannot, therefore, be attributed to any difficulty in detecting H^3 counts in the presence of C^{14}. (See also Fig. 3B.) In addition, it should be noted that only preparations of phage RNA, which show no evidence of degradation, yield this effect consistently.

The control preparations in Figs. 3A and 3B, to which RNA was not added, incorporated negligible amounts of either C^{14}-valine or histidine. There was the possibility that the system being employed discriminated against the synthesis of histidine-containing proteins because of some defect which was restored within the 6-min period. To check this eventuality, the experiment described in Fig. 3B was performed with the same extract as was employed in Fig. 3A. In this companion experiment, however, RNA message of *E. coli* was added. Since this is a heterogenous population of RNA molecules, initial preferential synthesis of a given protein type would not be expected. It is evident in Fig. 3B that no such difference exists in entry times of histidine and valine. The difference observed in Fig. 3A must, therefore, be ascribed to the viral RNA.

It would be expected from Fig. 3A that early samples would be characterized by a predominance of the non-histidine protein (peak 2). This expectation is realized as may be seen from the results in Fig. 4. Here, an incorporation with C^{14}-valine was terminated at 10 min and mixed with another sample which had been allowed to incorporate H^3-leucine for 60 min. The H^3 profile exhibits the presence of all three components. The C^{14} profile shows a strong peak 2, traces of peak 1, and little evidence of peak 3.

It was evident from our discussion of possible outcomes that the results described in Figs. 3A and 4 are inconsistent with any random translation of the cistrons in the viral RNA. Neither are they in agreement with an ordered translation with equal frequency for all cistrons. The data are most readily interpreted if the translating device performs its functions sequentially and, in so doing, makes significantly more product from the coat protein cistron than from the others. The fact that the appearance of the histidine-containing proteins is delayed suggests, that of the three corresponding to the major protein components, the coat protein is the first to be translated. It should be noted that the data do not eliminate the possibility that a cistron coding for a minor protein component may precede the coat protein cistron.

We come then to the rather surprising conclusion that it is indeed possible to exhibit evidence in a cell-free system for a type of translational regulation which is predictable from a numerical analysis of what occurs in the intact cell. In a polycistronic message, it determines the temporal order, and the number of times a given cistron is employed as a protein program. The experimental detection of this frequency regulation was made easier by the existence of polycistronic messages. It is clear, however, that the control of translation frequency can also be exerted on the messages containing only a single cistron.

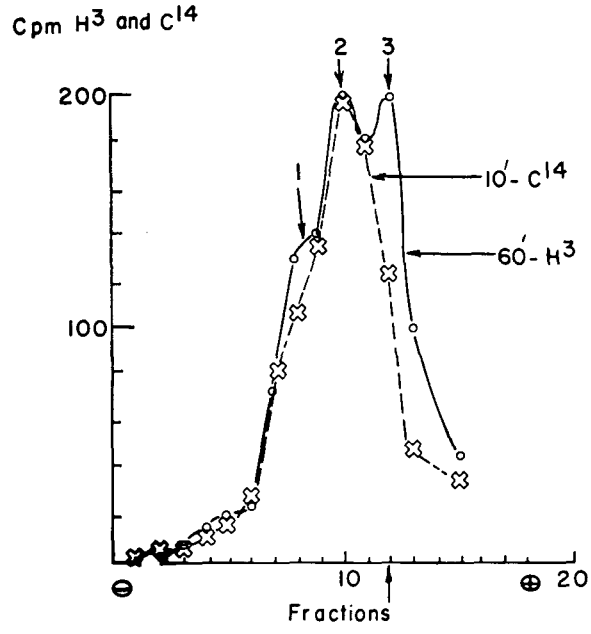

FIGURE 4. Incorporation for different time intervals. (Ohtaka and Spiegelman, 1963). The details of the conditions of the reaction are the same as described in Fig. 2. 0.2 ml of a reaction mixture incubated for 60 min at 35°C with H^3-leucine, and 0.1 ml of another reaction mixture incubated for 10 min at 35°C with C^{14}-valine, were mixed. They were adjusted to 5 M urea and .01 M EDTA and loaded on the column at the point indicated by the arrow. Electrophoresis and subsequent treatment for counting is as described in Fig. 2.

Various mechanisms can be proposed to account for the translational control devices detected. Although not the only one possible, the simplest way to explain the temporal order is to equate it to a corresponding linear sequential arrangement of the cistrons being translated. The frequency control might involve feedback inhibition by product, or some interaction between the component of the translating mechanism and a special sequence built into the message. This sequence may perhaps be related to the intercistronic genetic punctuation.

In any event, the results described make it evident that a further analysis of the molecular basis of translational regulation may now be obtainable in the comparative simplicity of a cell-free system. It seems plausible to imagine that this type of control is not confined to the RNA viruses,

but may also regulate the translation of normal polycistronic genetic messages. Translational control would, of course, operate in addition to the transcriptional devices to be discussed in succeeding paragraphs. A degree of flexibility is provided that might well have selective advantages. Thus, even if two cistrons generated messages in equal numbers. the corresponding proteins might be synthesized with very disparate frequencies.

F. RANDOM VERSUS NON-RANDOM TRANSCRIPTION OF A DNA GENOME

Recently accumulated data suggest that the transcription of an individual cistron into a ribopolynucleotide, and its subsequent translation into a polypeptide, are ordered and oriented processes. Thus, the ingenious genetic experiments of Crick et al. (1962) and Champe and Benzer (1962a) are readily interpretable if the reading of the base sequence starts from a fixed point and continues until the end of the cistron is indicated. Further, the elegant analysis of hemoglobin synthesis by Dintzis (1961) implies that the growth of a polypeptide chain is the result of the sequential addition of residues starting at the NH_2 terminal amino acid and finishing at the free carboxyl end.

Since every cistron is presumed to have its own beginning, each can in principle be read independently of the others. The experiments cited do not, therefore, provide an answer to the following question: *Is the transcription of the genome random or non-random?* By random we mean that the probability of transcription for any particular cistron is invariant with time and independent of its location in the genome.

The fact that specific proteins appear sequentially in phage infected bacteria (Luria, 1962; Kornberg et al., 1959; Dirksen et al., 1960, and Wiberg et al., 1962) does not answer the question since one can have an ordered use of randomly produced genetic messages.

An approach toward the solution of the problem may be seen by recognizing that, if the reading is random, then any sample of RNA messages produced in a synchronized system is equivalent to any other. If it is not random, a sample taken at one time should be distinguishable from that taken at another. To decide between these alternatives, three experimental conditions are required. One is a situation synchronized with respect to the onset in the transcription of a known genome. A second is a suppression of possible selective degradation of particular messages due to use in protein synthesis. The third is a method of differentiating the polynucleotide transcriptions of one set of cistrons from those of another.

The first and second requirements were readily provided by the *E. coli*-T2 system and the use of chloramphenicol. The third requisite was a little bit more difficult to achieve. To distinguish one type of RNA molecule from another, recourse was had to the methylated-albumin-kieselguhr (MAK) columns introduced by Lerman (1955) and subsequently developed and used by Mandell and Hershey (1960) to separate DNA from RNA and size fractionation of DNA. Others (Philipson, 1961; Otaka et al., 1962) have recently employed these columns to detect and isolate RNA molecules differing in size.

The discovery by Sueoka and Cheng (1962) that these columns can recognize base composition of DNA encouraged Kano-Sueoka and Spiegelman (in preparation) to see whether a similar recognition occurs with RNA. For present purposes, we may note two features of these columns which emerged from these investigations. They do fractionate RNA according to base composition. The higher the per cent of AU, the greater is the molarity of the sodium chloride required for elution. They also separate according to size with smaller fragments eluting at lower salt concentrations. Despite the fact that these two processes are occurring simultaneously, base compositional differences were readily detectable in different parts of the chromatographic profile of total T2 RNA. However, an increase in the resolution with respect to base composition can be obtained by prior fractionation with respect to size in linear sucrose gradients.

To employ such columns for the purposes we had in mind, and to insure a ready and sensitive detection of chromatographic differences, use was made of two identifying isotopic labels and simultaneous chromatography. Thus, consider two RNA preparations, one identified by H^3 and the other by C^{14}. If a mixture of the two is loaded on a column, the elution profiles of H^3 and C^{14} should be identical if the two preparations are the same and should differ if one contains one or more components absent from the other. To insure the comparability of RNA labeled in vivo for short periods of time, it is obviously necessary that the two labels be on the same precursor. One avoids, thereby, complications of varied pool sizes and different paths of entry. In the experiments to be described, C^{14} and H^3-uridine were used.

The plan of the experiment is obvious from the above discussion. Two aliquots of the culture are infected with T2, each is then allowed to incorporate one or the other of the labeled uridines for equal intervals at various times after the infection. The total RNA is then isolated and mixed in proportions determined by the specific activities of

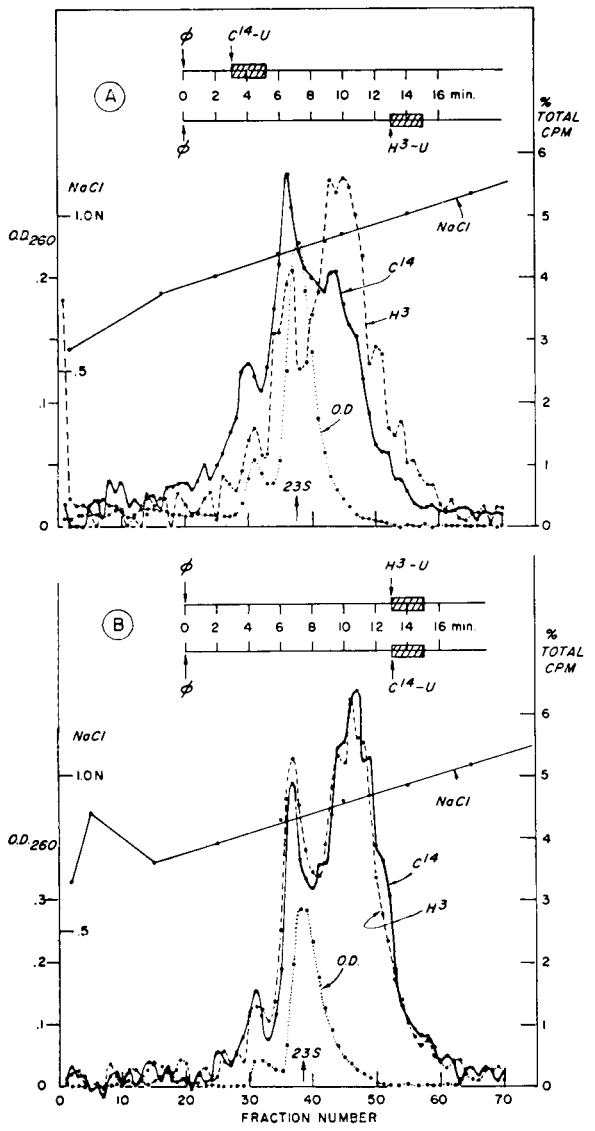

FIGURE 5. MAK column chromatography of T2-RNA. (Kano-Sueoka and Spiegelman, 1962).

(A) Mixture of two RNA samples; H^3-labeled coming from the culture pulsed between 13–15 min after infection and the C^{14}-labeled RNA derived from culture exposed to C^{14}-uridine between 3–5 min.

(B) Control mixture of two RNA samples independently labeled during the same period.

The protocol and timing of each experiment is indicated. ϕ indicates phage addition and CM chloramphenicol. The period of incorporation is indicated by the hatched rectangle.

the two isotopes. The mixture is then eluted from the column and the profiles of the two compared.

A typical experiment (Kano-Sueoka and Spiegelman, 1962) is summarized in Fig. 5, which compares the RNA synthesized in the same and in different periods. In every case of this experiment, chloramphenicol was added one minute prior to the addition of the radioactive uridine. The control experiment (B) of Fig. 5 compares two RNA preparations synthesized in the same time period. It is clear that the correspondence of the H^3 and C^{14} profiles is excellent. The agreement provides convincing evidence of the combined reproducibility of the various steps and operations required for such experiments. Figure 5A shows clearly that one can readily distinguish the RNA synthesized between 3 and 5 min from that which is formed between 13 and 15 min. This means that the mechanism which produced these two samples contains a non-random element. Consequently, the reading of the entire genome of T2 is a non-random process.

It should be noted that the methods developed for the present investigation possessed a degree of flexibility in resolving power which makes them useful for the experimental analysis of a number of problems and examples of these will appear in subsequent sections.

G. THE RELATION OF INDUCERS OF ENZYME SYNTHESIS TO TRANSCRIPTION

Historically, enzyme induction has played an early and continuous role in the development of our modern ideas on gene function. A little less than ten years ago it was shown definitively by tracer methodology (Rotman and Spiegelman, 1954; Hogness, Cohen, and Monod, 1955) that induction does, in fact, involve *de novo* synthesis of enzyme molecules. It was thus possible to state that the addition of an inducer such as thio-methyl-β-D-galactoside (TMG) to a wild type strain of *E. coli* specifically stimulates the synthesis of the enzyme β-D-galactosidase.

We now examine how this phenomenon can be analyzed with the concepts and experimental methods available to us now. Two sites of inducer action may be proposed in terms of the currently accepted view of information flow from the genome to the protein synthesizing machines. One site would be at the gene level, the inducer releasing an inhibition (Jacob and Monod, 1961) of the transcription mechanism that produces complementary RNA copies of the gene. The other site would be at the level of translation of the genetic messages into protein molecules. Although these alternatives are not mutually exclusive, their individual operation can be tested since techniques are available to provide an answer to the following question: *Does the presence of inducer increase the production of message homologous to the relevant genetic region?*

The detection and assay of RNA complementary to the genetic region responsible for β-galactoside utilization "lac" region can in principle be carried out by the DNA-RNA hybridization test (Hall and Spiegelman, 1961). A purified preparation of DNA

corresponding to the "lac" segment would furnish ideal hybridizing material for detecting the homologous RNA. An approximation is provided by the bacteriophage P1*dl*, a high-frequency transducing phage which has been shown to carry the "lac" operon (Luria et al., 1960; Franklin and Luria, 1961). The use of the DNA from this phage as a detecting device introduces some uncertainties due to possible homologies involving segments of the genome other than the "lac" region. Fortunately, the uncertainties introduced by extraneous homologies can be minimized by the device described in the preceding section of the two label co-chromatography procedure (Kano-Sueoka and Spiegelman, 1962). Thus, if induction stimulates message production, RNA unique to induced cells should be detectable by chromatographic fractionation. Further, the procedure should also distinguish "lac-specific" RNA from other RNA fractions that happen to be complementary to the detector DNA. Experiments along these lines were carried out by Hayashi, Spiegelman, Franklin, and Luria (1963). We briefly summarize the key experiments of this investigation.

The problem at hand can be answered using mixtures of RNA purified from the following three pairs of cultures. (A) Two inducible cultures, one

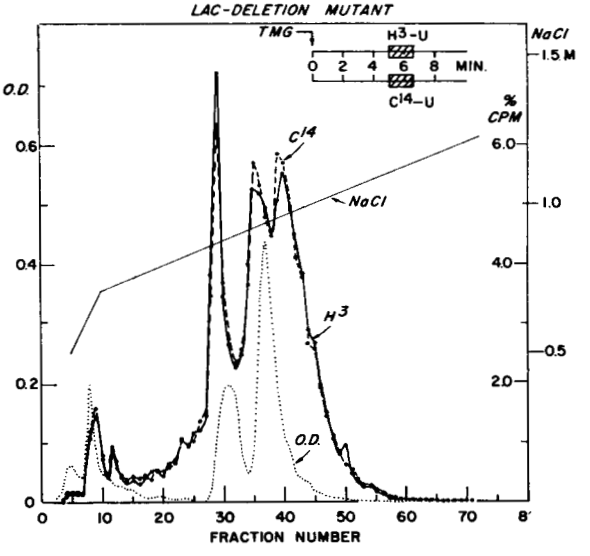

FIGURE 6. Column chromatography of RNA from cultures of "Lac"-deletion mutant with and without inducer. (Hayashi, Spiegelman, Franklin, and Luria, 1963).

As in all the other figures, protocols and timings are diagrammed. One culture was induced with IPTG at 0 time pulsed with H^3-uridine at 5 min. The other culture, without inducer, was pulsed with C^{14}-uridine. At 6.5 min incorporation was terminated; RNA was isolated from each culture and purified. A mixture of the two samples was chromatographed. OD profile identifies pre-existent stable components. NaCl gradient, measured by refractive index, is indicated. Here and in Fig. 7, counts are expressed as per cent of total to permit easier visual comparison of profiles.

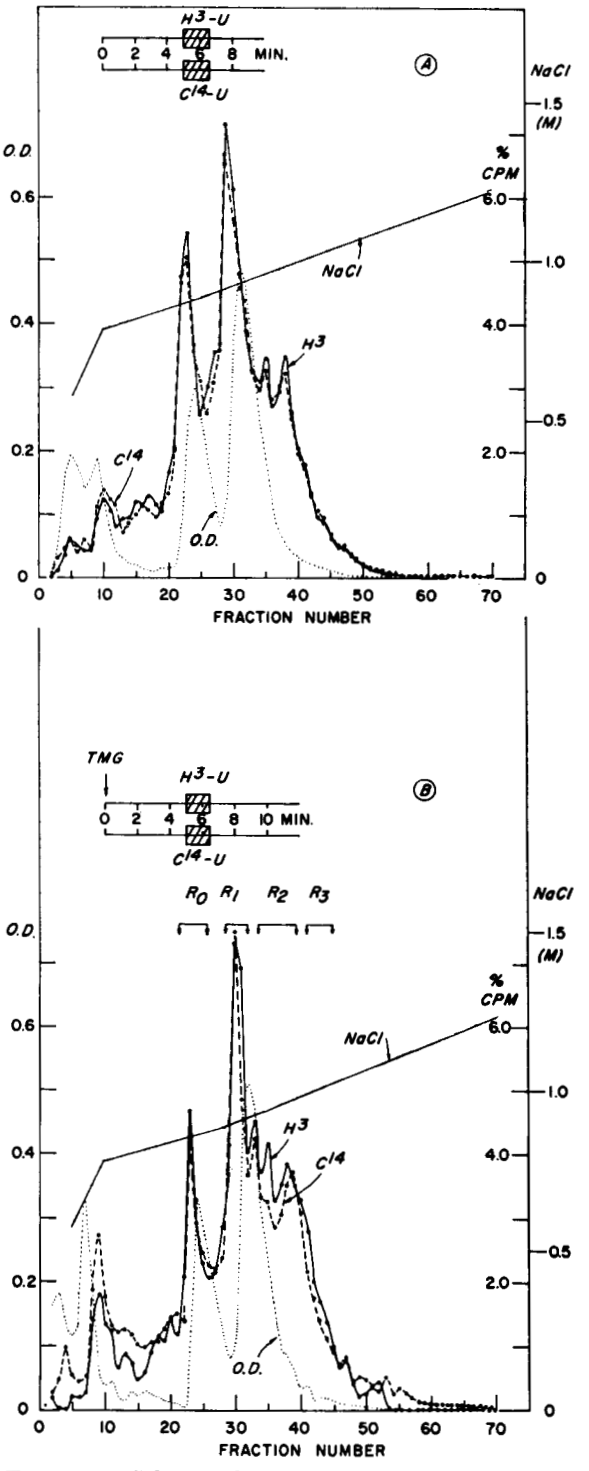

FIGURE 7. Column chromatography of induced and non-induced RNA from wild type. (Hayashi, Spiegelman, Franklin, and Luria, 1963).

(A) Control mixture of two RNA samples of *E. coli* BB independently labeled during the same period, without inducer.

(B) Mixture of induced and non-induced RNA samples. H^3 labels the induced culture, C^{14} the non-induced culture. All symbols and other details as in Fig. 6.

labeled with H^3-uridine in the presence of inducer, the other with C^{14}-uridine in its absence; (B) two inducible cultures, both without inducer, one labeled with H^3 and the other with C^{14}-uridine; (C) two "lac" deletion mutant cultures (W4032), one labeled with H^3-uridine in the presence of inducer, the other with C^{14}-uridine in its absence.

In mixture A, an inducer stimulated production of "lac" message would be signaled by the appearance in the H^3 profile of components which are absent in the C^{14} profile. These discordancies should not be present in the elution profiles of mixtures B or C. In mixture B neither culture contained inducer during labeling and it serves essentially to monitor the reproducibility of the profiles. Mixture C serves to detect irrelevant effects of inducer on RNA synthesis since in this instance the "lac" region is missing.

We first examine the two control preparations. Figure 6 describes the profiles of the RNA from the "lac" deletion mutants (mixture C). The presence of TMG causes no significant distortion in the distribution of one isotope with respect to the other. The excellent concordance of the C^{14} and H^3 in the other control, uninduced wild type (mixture B) is shown in Fig. 7A and proves that the various steps involved are reproducible in terms of the final outcome. Experimental mixture A is shown in Fig. 7B. Here we note two discordancies of the induced RNA (H^3 profile) in region R2. These are reproducibly observed whenever induced and non-induced RNA from an inducible strain are compared.

Direct evidence that these discrepancies correspond to messages from the "lac" region is provided by hybridization between P1*dl* DNA and various RNA fractions. To reduce the experiment to manageable proportions, fractions within the regions indicated in Fig. 7B as R_0, R_1, R_2, and R_3 were pooled, concentrated, and used in the hybridization tests. The RNase resistant counts found in the DNA density region in the cesium chloride gradient are then summed. Figure 8 summarizes the extent of the hybridization with P1*dl* and P1 DNA in terms of the cpm complexed in the different regions of the column effluent of Fig. 7B. The RNA components which hybridized with P1*dl* DNA appear to concentrate precisely in the region (R2) of the discrepancies observed between the induced and non-induced culture. They also appear to be fractionating at a different position from those components that can hybridize with P1 DNA. By correcting for hybrid formation with P1 DNA which does not contain a "lac" region, it appears that induction leads to at least a 30-fold increase in message RNA which specifically can hybridize with the "lac" operon. It must be emphasized that this is only a rough approximation and is very likely an underestimate by an order of magnitude.

The conclusions that can be drawn with respect to inducer function from these experiments and the similar ones by Attardi et al. (1962) with the "gal" locus may be briefly mentioned. It will be noted

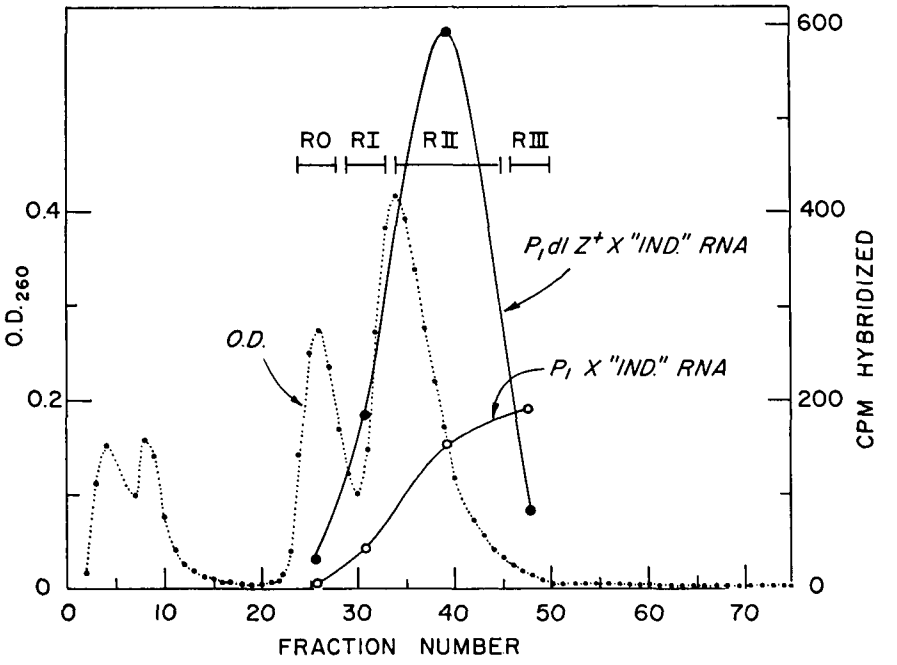

FIGURE 8. Column separation of "Lac" RNA and of RNA complementary to P1. (Hayashi, Spiegelman, Franklin, and Luria, 1963). For each region the amounts of RNA found to be hybridizable to P1 *dl* DNA and to P1 DNA are plotted. The OD profile is reproduced to provide a ready identification of relative positions.

that we have not considered the possibility that inducer functions by preventing message destruction. Such mechanisms are improbable in view of the cogent genetic arguments developed by Jacob and Monod (1961). Further, it must be recalled that the increases in "lac" RNA were observed in cultures that were actively synthesizing enzyme and presumably, therefore, using the corresponding message.

Any mechanism which would restrict inducer function to the site of message translation into protein is made unlikely by the present data. These findings do not eliminate, however, a double effect on both production and use of message. Neither do they specify that the primary site of inducer function is at the transcription step since this may be stimulated indirectly via a feedback mechanism, inactivation of repressor, or inhibition of its production. The experiments do indicate that whatever the detailed mechanism, the presence of inducer does indeed result in an increased amount of the corresponding transcriptions. They are consistent with the conclusion that the presence of inducer leads to an increased rate of transcription from the relevant genetic region. (See paper by Attardi, Naono, Rouvière, Jacob, and Gros in this volume for further experiments.)

H. DO ONE OR BOTH STRANDS OF THE DNA SERVE AS GENETIC MESSAGES?

At the 1961 Symposium the question of one or two strand transcription was raised (Brenner, 1961). There was an apparent correspondence between the base composition of the RNA message fraction and homologous DNA. The data were, however, not sufficiently numerous at that time to evaluate the degree of correspondence and the significance of discrepancies. In addition, it was pointed out (Spiegelman, 1961) that even good correspondence could be explained by either one or two strand transcription if the overall base composition of the two complementary strands are the same, or nearly so. The intervening two years has seen a resolution of this problem.

As it turned out, the first data to become available came from cell-free systems and these yielded an answer irrelevant to the normal situation. Several investigations (Geiduschek et al., 1961; Chamberlin and Berg, 1962; and Hayashi et al., 1963a) demonstrated that when double stranded DNA is employed as a template in vitro, the RNA dependent polymerase mediates the synthesis of RNA copies complementary to each of the two strands. Nevertheless, these investigations left open the question of whether this situation obtains in the intact cell.

In point of fact, accumulating evidence began to suggest that in vitro transcription may not involve both strands. Thus, on repeated examination, the base composition of unfractionated T2 complementary RNA (Volkin and Astrachan, 1956; Nomura et al., 1960) and the material collected by hybridization to DNA on columns (Bautz and Hall, 1962; Bolton and McCarthy, 1962) all have shown a persistent and significant inequality of guanine to cytosine. This discrepancy would be explained if only one strand is transcribed and that it has a bias toward a low C to G ratio. Furthermore, it has been shown (see sections B and C above) that sequences complementary to both ribosomal and soluble RNA exist in homologous DNA, implying that DNA generates these molecular species as well. Nevertheless, G does not equal C, nor does A equal U in ribosomal RNA, implying (Yankofsky and Spiegelman, 1962a) that transcription of the corresponding region of the genome does not involve both strands. A similar, although weaker, statement can be made for the soluble RNA cistrons. Finally, the ability of 5-fluorouracil to restore function in certain mutants of bacteriophage T4 also leads to the conclusion (Champe and Benzer, 1962b) that only one strand in the rII region yields RNA messages.

None of these findings are, however, decisive and they clearly do not define with certainty the details of the mechanism which reads the entire genome. The following three possibilities can be stated for genetic transcription. (1) All of both strands are transcribed into complementary RNA, (2) both strands are employed, but never in the same regions, (3) only one of the two strands serves as a template for genetic messages. The first is rather unlikely but we retain it for purposes of completeness. We are ignoring the possibility of regions which are not transcribed at all in either strand. This would introduce three more possibilities but they are not directly relevant to the central issue nor to the experiments to be described.

Examination of the possibilities mentioned suggest immediately that the hybridization test (Hall and Spiegelman, 1961) can be employed to provide data useful for deciding which of the three mechanisms operates in the cell. A successful resolution requires a source of radioactively labeled homologous complementary RNA and the availability of at least one of the two strands of the relevant DNA in pure form.

To attain the experimental requisites, attention was directed to the DNA virus ϕX174 which has been shown by Sinsheimer (1959) to contain only one of the two complementary strands. Nature has thus provided one of the necessary experimental components in an easily accessible form. However,

this useful feature can be fruitfully exploited only if the complementary strand can also be obtained. It has been shown (Sinsheimer et al., 1962) that immediately after infection, the complement to the injected strand is synthesized and a duplex results which has been called the "replicating form" (RF).

As a preliminary to any attempts to use this system, it was necessary to devise a procedure for the isolation in pure state of the RF-DNA of ϕX174 from infected cells. This was accomplished by Hayashi, Hayashi, and Spiegelman (1963a) through repeated chromatography on MAK columns. The RF-DNA was thereby isolated in greater than 95% purity. Its buoyant density in cesium chloride and melting temperature are characteristic for a double stranded DNA structure containing 43% guanine to cytosine. These data are precisely what would be expected if the RF-DNA consisted of one mature strand plus one corresponding to its complement.

In the same investigation this DNA was employed as a template for the transcribing enzyme to check whether the naturally occurring RF-DNA behaved as had been described for other double stranded DNAs. The nearest neighbors to uridylate were compared in the RNA synthesized when the RF-DNA and when the mature single stranded DNA were employed as templates in the in vitro system. The data showed that the mature DNA component in the replicating duplex does not serve as the sole source of complementary RNA. The results agreed best with the assumption that both strands of the replicating form function as templates. The data are therefore in agreement with all previous attempts to examine the question of strand copying in cell free systems.

The availability of both the RF and the single stranded DNA from mature virus particle in pure form made possible appropriate hybridization tests with the RNA message fraction of ϕX174. A few words must first be said about the conditions of message production in the infected *E. coli*-ϕX174 complex in order to understand the nature of the experiments which can be performed.

Investigation (Hayashi and Spiegelman, in preparation) of the ϕX174-*E. coli* complex quickly revealed that ϕX174 was incapable of shutting off macromolecular synthesis specific to the host cell. Thus, it was found that the RNA synthesized after infection was indistinguishable in overall base composition from that observed in uninfected cells. Further induced enzyme formation could be instituted for considerable periods subsequent to infection. We were faced, therefore, with the problem of detecting message production from ϕX174 in the midst of genetic messages generated

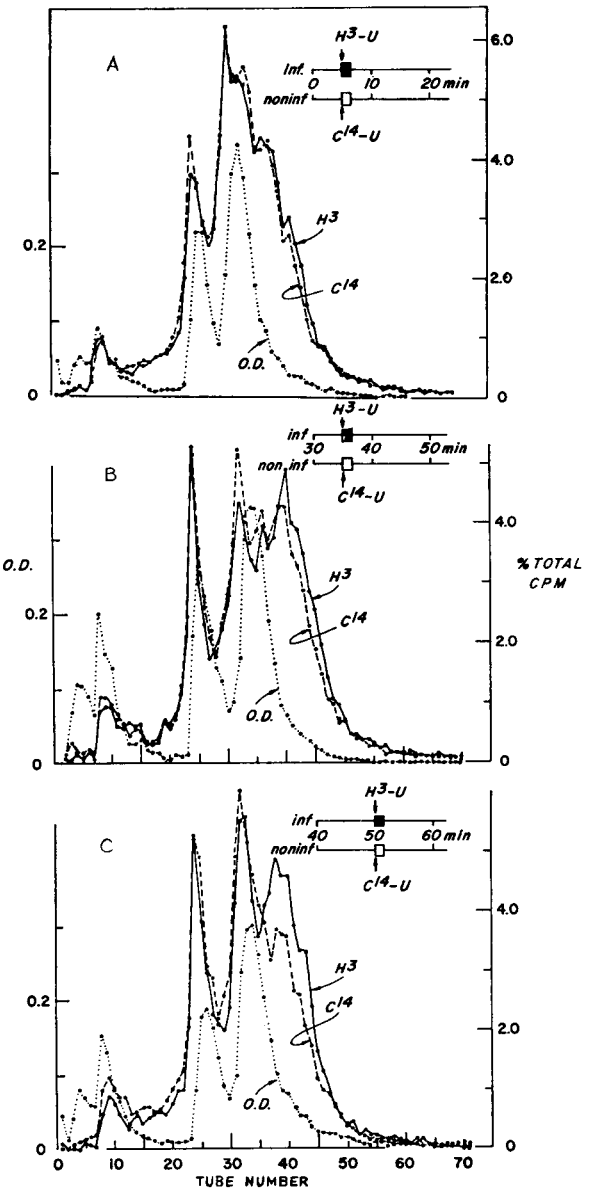

FIGURE 9. Detection of ϕX174 specific RNA by two-label co-chromatography on MAK columns. (Hayashi, Hayashi, and Spiegelman, 1963b). The protocol and timing of each experiment is diagrammed. Time is measured from 0 min (see text). A non-infected culture of *E. coli* was pulse-labeled with C^{14}-uridine for 90 sec. Infected cells were labeled with H^3-uridine at the same time and for the same period. On termination of incorporation, total RNA from both cultures was isolated and purified separately. A mixture of the two samples was chromatogrammed. The OD profile identifies pre-existent cellular stable components (16 S and 23 S indicated by arrows).

by the host genome. This is, however, readily achieved by the two-label and co-chromatographic procedure of Kano-Sueoka and Spiegelman (1962) already described.

To detect message specific to the genome of ϕX174, pairs of infected and non-infected cultures

were pulse-labeled with H^3-uridine in one case and C^{14}-uridine in the other. The RNA was purified from each and a mixture loaded on a MAK column. Figure 9 shows a series of the profiles obtained when such comparisons were carried out during different periods of infection. There is virtually no discrepancy between the profiles of H^3 and C^{14} in Figure 9A, suggesting that within 6.5 min little or no ϕX174 message has been produced in the infected cells. However, examination of the profiles obtained for the 35–36.5 min interval in Fig. 9B reveals discordancies unique to the infected complex. Finally, (Fig. 9C) the discrepancies between the H^3 and C^{14} become even more pronounced when the examination is extended to the 50–51.5 min period.

The greatest discordancies occur in the region to the right of the optical density peak corresponding to the 23 S ribosomal RNA. That these discrepancies are indeed referable to ϕX174 specific messages was tested by hybridization along the column using RF-DNA. A pulse similar to that carried out in Fig. 9C and the pooled material of the regions indicated in Fig. 10 were hybridized to RF-DNA. The amount of hybridizable RNA is indicated by the bar histogram. It is clear that there is a peak of RNA hybridizable to RF-DNA in the region corresponding to number 4.

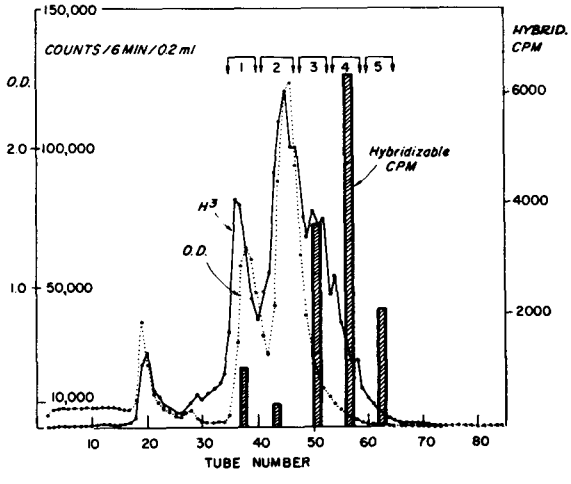

FIGURE 10. Column identification of ϕX174 specific RNA. (Hayashi, Hayashi, and Spiegelman, 1963b). ϕX174 infected *E. coli* culture was pulse-labeled with H^3-uridine for 90 sec, 50 min after infection. The total RNA was isolated and chromatogrammed. OD profile identifies pre-existing RNA. The pooled samples in the regions indicated in the figure were concentrated. The same number of counts from each sample was hybridized with 20μg of RF-DNA which had been heat-denatured in 1/10SSC (0.15 M na cl, 0.015 na citrate) at 97°–98°C for 15 min. Hybridization was carried out in 2 × SSC at 42.5°C for 18 hrs. The reaction mixture was chilled and 30 μg/ml of pancreatic RNase, free of contaminating DNase, was added. RNase treatment was performed at 26°C for 30 min. The reaction mixture was then loaded on an MAK column as described previously. Counts in the hybrid region were summed up and are shown in the bar-histograms.

From these and similar experiments one is led to conclude that prior to 5 min virtually all of the RNA messages found in the infected complex originate from the host genome. Viral specific messages accumulate later in the infection.

It may be noted in passing that because of the small size of ϕX-DNA, some of the procedures which have been useful in the past for detecting and accumulating RNA-DNA hybrids proved inconvenient. Cesium chloride density centrifugation in swinging bucket rotors (Hall and Spiegelman, 1961) yielded rather broad bands, particularly with heat-denatured material. Further, neither the DNA agar columns of Bolton and McCarthy (1962) nor the millipore filter techniques of Nygaard and Hall (1963) were found to be adequate for trapping this DNA. Consequently, a new procedure was devised (Hayashi, Hayashi, and Spiegelman, 1963b) which stemmed from the observation (Hayashi, Hayashi, and Spiegelman, 1963a) that single stranded DNA of ϕX174 is very well separated from its double stranded counterpart on MAK columns. RNA hybridized to DNA chromatograms in about the same position as the denatured DNA to which is it complexed.

The outcome of chromatographic examinations of three different hybridizing experiments is shown in Fig. 11. To identify the hybrid with certainty, both RF and mature single stranded DNA were labeled with P^{32} and the RNA with H^3. In all cases, the H^3-RNA employed was bulk RNA obtained by introducing H^3-uridine between 50–51.5 min after infection. It is clear from Figs. 11A and 11B that vegetative single stranded DNA, whether heated or not, cannot hybridize significantly to any of the H^3-RNA included in the reaction mixture. However, when heat-denatured RF-DNA is employed, one observes an RNase resistant tritiated component in the region of the single stranded DNA identified by the P^{32}. It was shown that the component in the complex carrying the isotope which identifies the RNA is alkali-labile and yields the expected 2′-3′ ribotides. These results imply ϕX174 RNA message is incapable of complexing with the single stranded DNA but can hybridize with the RF-DNA which contains both complementary components.

Table I summarizes a series of hybridization experiments carried out with labeled RNA derived from different periods of the infection. Comparatively little hybridization is observed between RF-DNA and RNA labeled between 5–6.5 min. This is in agreement with the chromatographic examination seen in Fig. 9. However, at later periods of infection the amount of hybridization observed with the

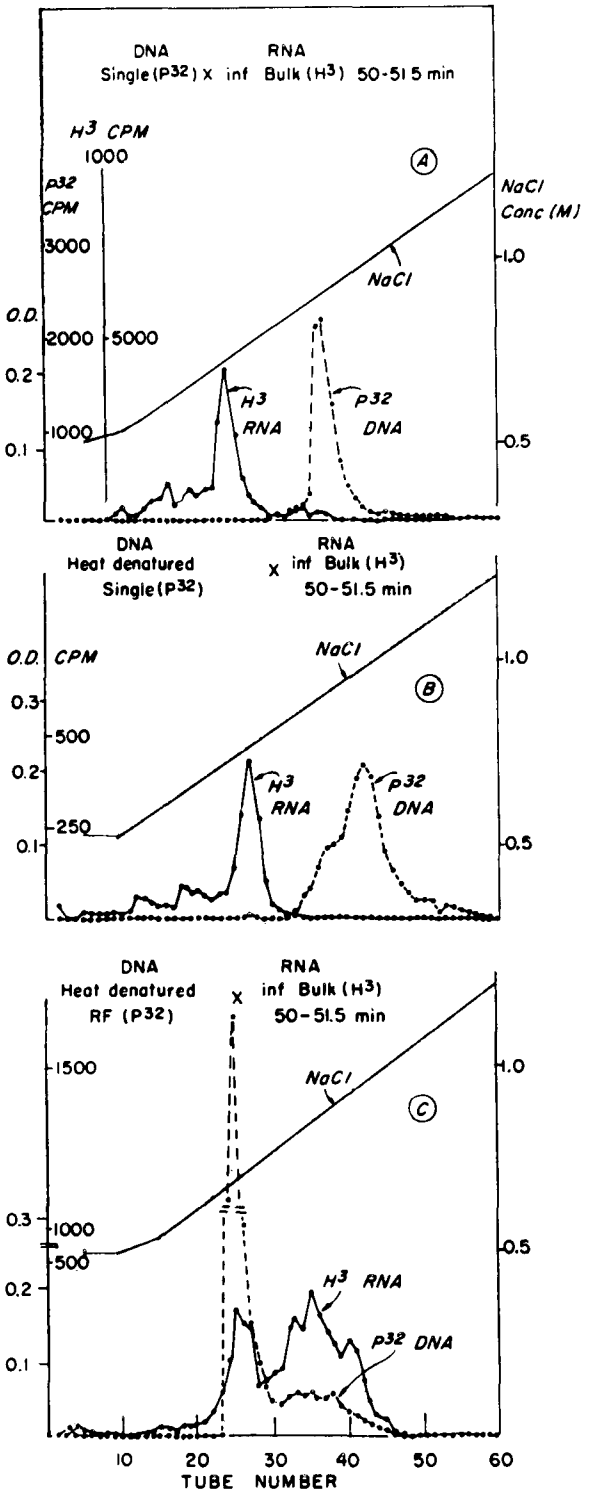

FIGURE 11. Hybrid separation by MAK column. (Hayashi, Hayashi, and Spiegelman, 1963b). 20 μg of RF or ϕX single DNA, both labeled with P^{32}, was hybridized with bulk RNA derived by introducing H^3-uridine between 50–51.5 min after infection. Hybridization, prior RNase treatment, and column procedure are as detailed in Fig. 10. Note that the RNase resistant core elutes earlier than the single stranded DNA and accompanying hybrid. By washing with NaCl of suitable concentration, the core can be removed completely from the column prior to the chromatographic separation of the hybrid structure.

TABLE 1. HYBRIDIZATIONS OF RNA WITH RF AND SINGLE STRANDED DNA
(Hayashi, Hayashi, and Spiegelman, 1963b)

RNA pulse labeled between (min)	DNA	
	RF	Single strand
5–6.5	253	<60*
35–36.5	3261	<160*
50–51.5	3473	<160*

* These represent upper limits estimated from summation over all tubes in the hybrid regions of the MAK column. The actual number is undoubtedly much lower.

20 μg of RF-heat denatured and 20 μg of single stranded DNA were hybridized with bulk RNA pulse-labeled with H^3-uridine in the intervals indicated in the first column. The conditions of hybridization, RNase treatment and subsequent isolation on an MAK column are as detailed in Fig. 10.

RF-DNA increases strikingly. In contrast, the single strand for the mature virus exhibits virtually no capacity to hybridize with RNA from any interval. The results in Table I agree, therefore, with the profiles observed in Fig. 11. Both indicate that ϕX174 messages are to be found in considerable amounts late in the infectious cycle and can hybridize only with the RF-DNA.

The fact that RNA messages produced in the *E. coli* ϕX174 complex hybridize effectively only with RF-DNA offers strong support for the assertion that the component of the RF-DNA which serves as a template for message production corresponds to the complement of the DNA strand found in the vegetative particle. To complete the proof, it was necessary to demonstrate that the base composition of the RNA hybridized to RF-DNA corresponds to that which this assertion predicts, i.e., the complexed RNA should be complementary to the complement of the mature vegetative strand. As a consequence, it should possess a base composition which mimics that of the vegetative DNA. The numerical situation is such as to make an experimental test of this prediction easily obtainable.

The necessary P^{32}-labeled RNA was prepared by 3 min labeling of the injected complex at various times. As an added precaution, a simple device was employed (described in Table 2) to make a preparation which would provide an adequate sample of all ϕX174 messages. The labeled RNA thus obtained was hybridized to RF-DNA and the hybrid mixture subjected to RNase. The product was then chromatogrammed on MAK columns and the hybrid region collected, hydrolyzed with alkali, and the base composition of the labeled nucleotides determined. The results are summarized in Table 2. The base composition of the RNA hybridized

TABLE 2. BASE RATIO OF HYBRIDIZABLE RNA
(Hayashi, Hayashi, and Spiegelman, 1963b)

	C	A	U(T)	G
RNA				
all stage pulsed (0–54 min)	17.5	23.8	33.1	25.6
short pulse (50–53 min)	17.5	25.5	34.0	23.0
DNA				
ϕX174 single*	19	25	33	23
ϕX174 complementary†	23	33	25	19
ϕX174 RF‡	21	29	29	21

* Taken from Sinsheimer (1959).
† Assumed complementary to the original strand.
‡ Assumed double stranded with single and complementary strands.

E. coli C in log phase was concentrated to 10^{10}/ml in AD. (Tris-Hcl 0.05 M pH 7.3; 10^{-3} M phosphate; 10^{-3} M $MgSO_4$; 0.05% casein hydrolysate; 1 μg/ml of $FeCl_3$; 10^{-3} M $CaCl_2$; 10^{-2} M NaCl; 10^{-2} M KCl). ϕX174 was added at m.o.i. $\sim$ 20. For the complete message sample, adsorption of the phage was performed at 20°C for 30 min, 1 ml of this complex was added every 3 min into prewarmed SCXD (Tris-Hcl 0.05 M pH 7.3; 0.5% glycerol; 10^{-3} M phosphate; 10^{-3} M $MgSO_4$; 0.05% casein hydrolysate, 1 μg/ml $FeCl_3$); (150 ml) at 30°C under aeration. When the 18th complex was added (51 min after 0 time), 20 mc of P^{32} was pulsed for 3 min. The RNA was then isolated, purified, and designated as "all stage-pulsed" RNA. A short P^{32} pulse between 50–53 min after infection was also performed and RNA was isolated and purified.

6 mg of each bulk RNA was hybridized with 70 μg of heat-denatured RF; hybridization, RNase treatment are as detailed in Fig. 10. The hybrid was isolated on a MAK column and the base composition determined.

coming from the 50–51.5 min period is in good agreement with that obtained from the sample expected to contain equivalent amounts of all messages synthesized during the entire period of the infection. Comparison of the three DNA base compositions listed reveals that the hybridized RNA is similar to the vegetative single strand and is complementary to its complement. The prediction that the complement to the mature strand generates the genetic messages appears to be confirmed.

All of the results reported are consistent with the inference that one of the two strands of the DNA duplex is predominantly, or solely, used to generate genetic messages. This conclusion does not deny the possibility that effective transcription of only one of the strands requires the presence of both.

It may perhaps be proposed that deductions derivable from the study of a single stranded DNA virus may not be generally applicable to organisms which normally possess both complementary strands. However, it must be noted that message production does not begin in this system until the double stranded structure is constituted. One is inclined, therefore, to believe that the situation being examined is not so abnormal as to be completely unique. Nevertheless, general acceptance obviously requires confirmation with other DNA systems.

The fact that only one strand is used possesses an interesting implication for the problem of genetic inversions. Sequence inversion in a DNA molecule requires, in addition, a 180° rotation around the axis of the helix, in order to reconstitute the antiparallel 5'-3' internucleotide linkage. This exchanges sequences between the strands. Thus, inverted sequences will be lost to the transcription mechanisms so that inversion necessarily results in a deletion. One would predict, therefore, that transcribable inversions leading to non-deletion phenotypes will not be observed in organisms which contain a continuous DNA duplex structure as the sole component of their chromosomal apparatus. The corollary of this for organisms which do exhibit non-deletion type inversions is obvious. (The desirability for extending these conclusions to DNA molecules which are double stranded in the vegetative state has already been met; see J. Marmur and G. Toccini-Valentini et al., in this volume. See also W. B. Wood and P. Berg; and M. Chamberlin and P. Berg for important related observations on transcription.)

SUMMARY

The present paper records our efforts over the past two years to clear up some of the unresolved issues of information transfer employed by DNA.

It will be noted that two experimental devices have been found to be repeatedly useful. The DNA-RNA hybridization test was converted into a sensitive and powerful tool by the use of isotopic labeling and the RNase resistance of hybrid structures. By this means it became possible to search for, and detect, sequences corresponding to as little as 0.01% of an *E. coli* genome. The other technique involved the "two-label simultaneous chromatographic method" which permits the detection and identification of genetic messages generated by particular loci. With the aid of these, and some auxiliary devices, it was possible to perform informative experiments which advanced our understanding of the following interesting problems:

1. ORIGIN OF THE TWO RIBOSOMAL RNA COMPONENTS.

It was shown that homologous DNA contains sequences complementary to the 23 S and 16 S ribosomal RNA molecules. Further, it could be shown by competition experiments that the two are derived from DNA sequences unique to each. By saturation curves it could be estimated that the 23 S cistrons occupied about 0.2% of the genome and the 16 S, 0.1%.

2. The Origin of S-RNA.

Hybridization tests showed that DNA contains sequences complementary to homologous S-RNA. About 0.02% of the *E. coli* genome is employed for this purpose, suggesting that there are approximately 2.5 stretches for each of the 20 amino acid classes of S-RNA.

3. Polycistronic Messages and "Operon" Operation.

The existence of genetic messages too large to code for a single protein suggested the existence of RNA molecules which coded for several proteins. This provided a simple molecular basis for the mechanism of operon functioning. Thus, it could be postulated that cistrons belonging to the same operon would be transcribed into one continuous RNA molecule. The existence of such "polycistronic" messages raised obvious possibilities of regulation at the point of their translation into protein. Such controls could determine the order of and frequency with which the component cistrons are used as protein programs.

Translational regulation would of course operate, in addition to the controls at the level of transcription. A degree of flexibility would thereby be provided which might well be advantageous. Thus, even if two loci generated precisely equal numbers of messages the corresponding proteins might be synthesized with very disparate frequencies.

4. Order and Frequency Control in the Translation of a Polycistronic Message into Protein in vitro.

Viral RNA was employed as the obvious experimental choice to analyze the functioning of polycistronic messages. Such RNA molecules must be programs for at least two proteins, and they clearly provide an ideal source of a uniform population, each of which codes for the same limited number of protein molecules. Using the RNA from the RNA bacteriophage MSϕ2, it was shown that at least three electrophoretically separable proteins were synthesized in a cell-free system. One was the coat protein which lacks histidine. Comparison of the kinetics of the appearance of histidine and non-histidine containing proteins provided evidence for the existence of a control mechanism which determines the order and frequency of translation of each cistron.

5. Random versus Non-Random Transcription of the Genome.

The T2-*E. coli* complex was used as a convenient source of a synchronized onset in the transcription of a known genome. The use of the two label co-chromatographic procedure permitted an unambiguous decision between differences and similarity of any two samples. They were always easily distinguishable if they were synthesized at different times and identical if they came from the same time period. This is consistent with an ordered transcription of the genome.

6. On the Mechanism of Inducer Action.

Hybridization of RNA with DNA from a transducing phage carrying the "lac" genetic region and the double label chromatographic fractionation of RNA were employed to detect the specific "lac" message RNA of *E. coli*. The experiments demonstrated the occurrence in induced cells of increased amounts of RNA complementary to the "lac" region. This RNA can be identified chromatographically and distinguished from other *E. coli* messages which possess homology for some portions of the phage genome. The data obtained are consistent with the conclusion that the presence of inducer leads to an increased rate of transcription from the "lac" region.

7. Restriction of Transcription to One of the Two Complementary Strands of DNA.

To settle the question of whether one or both DNA strands are used to generate genetic messages, recourse was had to ϕX174, a single stranded DNA virus.

The experiments used the DNA from the mature virus particle and the purified "replicating form" DNA, which contains the original strand and its complement. Appropriate hybridization tests with these two DNA preparations and the RNA message fraction were carried out. The results of both the hybridization tests and of the base compositional analysis of the RNA complexed revealed the presence of RNA complementary to only one of the two strands of the RF-duplex. The data are consistent with the conclusion that only one of the two complements in a DNA duplex is either the principal, or sole source, of utilizeable genetic information. These results further imply that transcribable inversions leading to non-deletion phenotypes will not be found in organisms containing a continuous DNA molecule as the sole component of their chromsomal apparatus.

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